

Purification and Characterization of Avian β -Defensin 11, an Antimicrobial Peptide of the Hen Egg[∇]

Virginie Hervé-Grépinet,¹ Sophie Réhault-Godbert,¹ Valérie Labas,² Thierry Magallon,²
Chrystelle Derache,³ Marion Lavergne,¹ Joël Gautron,¹
Anne-Christine Lalmanach,³ and Yves Nys^{1*}

INRA, UR83 Recherches Avicoles, F-37380 Nouzilly, France¹; UMR INRA 85, CNRS 6175, Université François Rabelais, Physiologie de la Reproduction et des Comportements, Plate-forme de Protéomique Analytique et Fonctionnelle, F-37380 Nouzilly, France²; and INRA, UR 1282 Infectiologie Animale et Santé Publique, F-37380 Nouzilly, France³

Received 12 February 2010/Returned for modification 5 April 2010/Accepted 17 June 2010

Natural antimicrobial peptides are present in different compartments (eggshell, egg white, and vitelline membranes) of the hen egg and are expected to be involved in the protection of the embryo during its development and to contribute to the production of pathogen-free eggs. In the present study, we used vitelline membranes from hen (*Gallus gallus*) eggs as a source of avian β -defensin 11 (AvBD11). A purification scheme using affinity chromatography and reverse-phase chromatography was developed. Purified AvBD11 was analyzed by a combination of mass spectrometry approaches to characterize its primary sequence and structure. A monoisotopic molecular species at $[M + H]^+$ of 9,271.56 Da was obtained, and its N- and C-terminal sequences were determined. We also examined posttranslational modifications and identified the presence of 6 internal disulfide bonds. AvBD11 was found to exhibit antimicrobial activity toward both Gram-positive and Gram-negative bacteria.

The avian egg is a unique and original biological system which is formed according to a well-defined spatial and temporal sequence as it passes along the reproductive tract of the hen. The segments of the oviduct involved in the egg formation are the infundibulum, the magnum, the isthmus, and the uterus. Each segment expresses and secretes specific molecules that become successively incorporated into the vitelline membranes in the infundibulum, the egg white in the magnum, the eggshell membranes in the isthmus, and the eggshell in the uterus. Thus, the hen deposits into the egg, which can be considered a closed chamber, all the nutrients and protective systems that are necessary to support the development of an embryo during 21 days of incubation. Recently, transcriptomic and proteomic approaches have identified almost 1,000 putative proteins and peptides in the various compartments of eggs (6). Among these are a number of polypeptides that are likely to resist microbial contamination of the eggs, some of which belong to the family of avian β -defensins (AvBDs).

A total of 14 avian β -defensin genes have been identified through *in silico* studies. Because most of them were simultaneously described by two research groups, some confusion was generated due to different nomenclatures (9, 10, 18, 31). In a collaborative venture, we proposed a novel nomenclature that adopted the numbering system used by Xiao et al. (31) and replaced the term “gallinacin,” used formerly by Lehrer’s group, with “avian β -defensin” (AvBD) (19). AvBDs are small cationic nonglycosylated peptides (1 to 9 kDa). Some of them were successfully tested for their antimicrobial activity, includ-

ing chicken AvBDs AvBD1, -2, -7, and -9, turkey AvBD1 and -2, ostrich AvBDs AvBD1, -2, -7, and -8, and penguin AvBD103b (3, 30). In mammals, β -defensins are molecules of innate and adaptive immunity, with a broad spectrum of antimicrobial activity (1). β -Defensin molecules possess six highly conserved cysteines with the following consensus sequence motif, where C is a cysteine, G a glycine, and X any amino acid: $X_n-C-X_{2-4}-G-X_{1-2}-C-X_{3-5}-C-X_{9-10}-C-X_{5-6}-CC-X_n$. The six cysteines in β -defensins are linked to form disulfide bridges in a 1–5, 2–4, and 3–6 pairing pattern (5).

Little is known regarding the tertiary structure of β -defensins in avian species. Only the three-dimensional structure of the king penguin avian β -defensin 103b, (AvBD103b or spheniscin-2) has been solved recently. The tertiary structure of AvBD103b is very similar to those of mammalian β -defensins, including the common array of disulfide bonds and the presence of a three-stranded β -sheet as the main secondary structure element (15, 26). AvBD103b was isolated from the stomach contents of the king penguin and exhibits a wide spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi (29). The high potency of AvBD103b against bacteria was correlated with its extreme cationicity but also with the presence of a hydrophobic patch which is not present in mammalian β -defensins (15).

AvBDs are expressed in several tissues, including the genital tract, where they are thought to play a role in the antimicrobial defense of the hen genital tract. This protection is essential for embryonic development and also ensures that the *Gallus gallus* table egg is free of pathogens. AvBD1, -2, -3, -4, -5, -8, -9, -10, -11, and -12 mRNAs have been shown to be expressed at moderate levels in the infundibulum, the magnum, the isthmus, the uterus, and the vagina of the laying-hen oviduct (20). A recent study reported that primary epithelial cell cultures of the laying-hen oviduct constitutively expressed most AvBDs,

* Corresponding author. Mailing address: INRA, UR83 Recherches Avicoles, Fonction et Régulation des Protéines de l’Oeuf, F-37380 Nouzilly, France. Phone: 332 47 42 72 82. Fax: 332 47 42 77 78. E-mail: Yves.Nys@tours.inra.fr.

[∇] Published ahead of print on 12 July 2010.

with the exception of AvBD2, -6, -7, and -8, at moderate to high levels. All these studies showed the mRNA expression of a large panel of AvBDs in the different regions of the laying-hen oviduct, suggesting that they might together exhibit a very large spectrum of antimicrobial activity. Surprisingly, in spite of the expression of AvBD1, -2, -3, -4, -5, -8, -9, -10, -11, and -12 mRNAs in the different segments of the oviduct, only three AvBDs have been identified in the hen egg by proteomic approaches, AvBD11 in the eggshell, the egg white, and the vitelline membranes, AvBD10 in the eggshell, and gallin, related to the AvBD family (8), in the egg white (21–23).

AvBD11 corresponds to a molecule initially identified as vitelline membrane outer layer protein 2 or VMO-2 (21–23), one of the four major proteins in vitelline membranes along with ovomucin, lysozyme, and VMO-1 (11, 27). VMO-2 was first described as a molecule of 53 amino acids, a molecular mass of 6,000 Da, high cysteine content, four disulfide bonds, and LPRDTSRXVGYHGYXIRSKV as the amino-terminal sequence (12). In 2004, an *in silico* approach, based on the chicken genome, predicted that AvBD11 possessed 80 amino acid residues, with RDTSRCVGYHGYCIRSKVCP as the N terminus and an expected monoisotopic molecular mass of 9,078.56 Da for the mature peptide (31).

In the present work, we confirm the identification of mature AvBD11 peptide in the hen egg and describe its purification from vitelline membranes. Using bottom-up and top-down proteomic approaches, we redefine its primary structure, as well as confirming the N- and C-terminal sequences of the mature peptide and its posttranslational modifications. Additionally, the antimicrobial activity of AvBD11 is characterized against different bacterial strains and compared to that of the king penguin avian β -defensin, AvBD103b.

MATERIALS AND METHODS

Preparation of polyclonal anti-AvBD11 antibodies. The N- and C-terminal extremities (RDTSRCVGYHGYC and GLCPKRWTCCKEL, respectively) of AvBD11 were synthesized and conjugated with keyhole limpet hemocyanin (KLH) (Genosphere Biotechnologies, Paris, France). Sera against AvBD11 were prepared as follows. Two rabbits were immunized four times at 3-week intervals by intramuscular injection of 500 μ g of the synthesized extremities of AvBD11 bound to KLH and emulsified in 50% complete Freund adjuvant for the first injection and in 50% incomplete Freund adjuvant for the next three injections. The rabbits were euthanized 3 weeks after the last injection. The blood was allowed to clot at room temperature for 2 h, stored overnight at 4°C, and then centrifuged at 2,000 \times g to remove blood cells. Sera were collected and stored at –20°C.

Vitelline membrane preparation. Vitelline membranes were obtained from unfertilized Isa Brown eggs. Following separation of the egg white from the yolk, the vitelline membrane was punctured to evacuate the yolk. After several successive washings in distilled water, the membranes were dried, lyophilized, and frozen in nitrogen liquid to be crushed very finely. The vitelline membrane proteins were solubilized in 50 mM Tris-HCl, 0.5 M NaCl, pH 7.4. After a first sonication and a gentle overnight agitation at 4°C, one additional sonication was performed. Samples were then centrifuged for 10 min at 10,000 \times g at 4°C. The supernatant was collected, and the protein concentration was determined using a Protein Dc Assay (Bio-Rad, Marnes-la-Coquette, France), with bovine serum albumin (BSA) (Interchim, Montluçon, France) as the standard, according to the manufacturer's instructions.

Heparin-Sepharose chromatography. Heparin-Sepharose chromatography was performed using the batch method, following the manufacturer's instructions. Briefly, the solubilized proteins of the vitelline membrane were incubated with heparin-Sepharose beads in 50 mM Tris, 150 mM NaCl, pH 7.4, overnight at 4°C under constant shaking. The beads were washed extensively with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, until the absorbance at 280 nm reached zero and then loaded onto a polypropylene column (Qiagen, Courtaboeuf, France).

The elution of bound proteins was achieved using 50 mM Tris-HCl, 1 M NaCl, pH 7.4. The protein concentrations of unretained (unbound) and eluted (bound) fractions of the vitelline membranes were determined using a Protein Dc assay as described above.

Gradient SDS-PAGE. Equal amounts of total proteins (total vitelline membrane proteins and eluted or unretained fractions from heparin-Sepharose beads) were denatured in 5 \times sodium dodecyl sulfate (SDS) sample buffer (0.31 M Tris-HCl, pH 6.8, 20% SDS, 25% glycerol, 0.01% bromophenol blue, 25% 2- β -mercaptoethanol) and boiled for 5 min. Proteins were separated on one-dimensional 4-to-20% gradient SDS-PAGE (SDS-polyacrylamide gel electrophoresis) GeBaGel gels according to the manufacturer's instructions (BioScience Innovations, Interchim, Montluçon, France), and the gels were stained with Coomassie brilliant blue R250.

Western blotting. Proteins were transferred to nitrocellulose membranes after migration on the 4-to-20% GeBaGel gels. The nitrocellulose membranes were blocked for 1 h in 5% Blotto (Bio-Rad, Saint-Quentin, France) in phosphate-buffered saline (PBS) and incubated overnight at 4°C with anti-AvBD11 antiserum (1/1,000) in PBS, 2% Blotto. After further washes in PBS, 0.1% Tween 20, the membranes were incubated for 90 min at room temperature with anti-rabbit IgG secondary antibody coupled to Alexa 680 (Molecular Probes, Eugene, OR) that was diluted 1/2,500 in PBS, 2% Blotto. Immunoreactive bands were detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

In-gel digestion and protein identification by nano LC-ion trap mass spectrometry. Protein bands were excised from the gel, rinsed with water and acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide, and then incubated overnight at 37°C in 25 mM NH_4HCO_3 with 12.5 ng/ μ l trypsin (sequencing grade; Roche, Paris) as described by Shevchenko et al. (28). The tryptic digestion fragments were extracted, dried, and reconstituted with 0.1% formic acid before being sequenced by nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS-MS). The Ettan MDLC system (GE Healthcare, Germany) was used for desalting and separation of tryptic peptides prior to online MS and MS-MS analyses. Ten microliters of digested sample were injected and automatically desalted and preconcentrated using a 300- μ m (inner diameter) by 5-mm Zorbax 300-SB C_{18} trap column (Agilent Technologies, Germany). Peptide separations were conducted on a 75- μ m (inner diameter) by 150-mm Zorbax 300-SB C_{18} column (Agilent Technologies, Germany). Buffer A consisted of water with 0.1% formic acid, while buffer B was 84% acetonitrile with 0.1% formic acid. Separation was performed at a flow rate of 350 nl/min by applying a gradient of 15-to-55% buffer B over 60 min. The eluted peptides were analyzed online with an LTQ linear ion trap mass spectrometer (Thermo Electron, United States). Each scan cycle consisted of one full-scan mass spectrum (m/z , 500 to 2,000) collected in enhanced mode followed by three MS-MS events in centroid mode. For collision-induced dissociation (CID) spectra (MS-MS), the isolation width was 2 m/z units and the normalized collision energy was 40%. Dynamic exclusion was activated for 30 s with a repeat count of 1. The raw data files were converted to mzXML with Bioworks 3.3.1 software (Thermo Fischer Scientific, San Jose, CA). In order to identify the proteins, the peptide and fragment masses obtained were matched automatically against a locally maintained copy of the NCBI nr database (downloaded 2 February 2009). MS-MS ion searches were performed using Mascot Daemon and Mascot's search engine (Matrix Science, United Kingdom) against the *Chordata* section (7,873,120 sequences). The enzyme specificity was set to trypsin with 2 missed cleavages using carbamidomethylcysteine and methionine oxidation as variable modifications. The tolerance of the ions was set to 1.4 Da for parent and 1.0 Da for fragment ion matches. Proteins detected with a P value of <0.05 were considered positively identified with one peptide when the presence of 5 consecutive fragment ions was confirmed.

Reverse-phase HPLC. Proteins present in heparin-Sepharose-eluted fractions were further separated by preparative X-Bridge C_{18} (Waters) reverse-phase high-performance liquid chromatography (RP-HPLC). A linear 5-to-65% acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) was used at a constant flow rate of 0.90 ml/min during 85 min. The content of each collected peak was analyzed by matrix-assisted laser desorption-ionization (MALDI-TOF) mass spectrometry.

MALDI-TOF mass spectrometry. All mass spectra were generated on a M@LDI LR (Micromass [Waters] Ltd., Manchester, United Kingdom) MALDI-TOF mass spectrometer operating in the positive linear mode. Mass spectra were recorded in the m/z range of 2,000 to 20,000, acquiring 10 shots per spectrum at a laser firing rate of 10 Hz. Data processing was performed using MassLynx 4.0 software. The background of the spectrum result for each sample well was subtracted using a polynomial order of 10% below the curve and smoothed with the minimum peak width at half height set to 15 channels. Smoothing was performed twice using the Savitzky Golay algorithm. All the spectra were pro-

cessed using the same parameters. For mass fingerprint analysis of reverse-phase HPLC-eluted peptides, 1 μ l of each fraction and the matrix (1:1 [vol/vol]) were loaded onto the target by using the dried droplet method. The matrix used was sinapinic acid at 20 mg/ml dissolved in 50% acetonitrile–50% H₂O–0.1% TFA.

In-solution digestion and identification by nano LC-Q-TOF mass spectrometry. Molecular species purified by reverse-phase HPLC were identified after in-solution tryptic digestion. Ten microliters of purified peptides were mixed in 10 μ l of 50 mM NH₄HCO₃ and then reduced with 5 mM dithiothreitol, alkylated with 12.5 mM iodoacetamide, and incubated overnight at 37°C with 0.5 μ g of bovine trypsin (sequencing grade; Roche, Paris, France) and 1 μ l of ProteaseMax surfactant (Promega, Charbonnière-les-Bains, France). The tryptic digests were incubated for 1 h at 60°C, acidified with 10 μ l of 5% trifluoroacetic acid, and then sonicated for 10 min. Nanoscale capillary liquid chromatography–tandem MS (nano LC–MS–MS) analysis of the digested peptides was performed using a CapLC system coupled to a hybrid quadrupole-time of flight (Q-TOF) Ultima Global mass spectrometer (Waters, Manchester, United Kingdom). Samples were desalted and concentrated using an online precolumn (monolithic trap column, 200 μ m [inner diameter] by 5 mm; Dionex/LC Packings, Voisins-le-Bretonneux, France). Peptide separations were conducted on two online analytic columns (Pepswift monolithic nanocolumn, 100 μ m [inner diameter] by 50 mm; Dionex/LC Packings, Voisins-le-Bretonneux, France, and Silica Nanocolumn Strategy, 75 μ m [inner diameter] by 500 mm; Interchim, Montluçon, France) running with a 200-nl/min flow. The gradient profile consisted of a gradient from 100% buffer A (0.1% formic acid–2% acetonitrile–98% H₂O [vol/vol]) to 10% buffer B (0.1% formic acid–20% H₂O–80% acetonitrile [vol/vol]) in 1 min and from 15% to 60% buffer B in 120 min. Mass data were acquired using automatic switching between MS and MS-MS (fragmentation) modes: one MS survey scan was followed by MS-MS scans on the four most intense peptide ions detected. Only doubly and triply charged ions were allowed to be selected as precursors over an m/z range of 400 to 1,300. The data were processed using ProteinLynx Global server 2.2 (Waters), and the peak list was exported in pkl file format. In order to identify the proteins, the precursor and fragment masses obtained were matched automatically against a locally maintained copy of the NCBI nr database (downloaded 2 July 2009) in the *Chordata* section (9,244,709 sequences), using the MS-MS ion search option in Mascot, version 2.2.04 (Matrix Science, United Kingdom). The enzyme specificity was set to semitrypsin with four missed cleavages, using carbamidomethylcysteine and methionine oxidation as variable modifications. The tolerance of the ions was set to 0.1 Da for both parent and fragment ion matches. All hits with P values of <0.05 were manually verified to confirm the presence of five consecutive fragment ions.

Structural characterization of intact AvBD11 by nano ESI-Q-TOF mass spectrometry. Five microliters of each HPLC fraction was diluted with 10 μ l of a mixture of formic acid 1%–acetonitrile (1:1) and analyzed by MS and MS-MS on a nanoelectrospray ionization (nano ESI)-Q-TOF Ultima Global mass spectrometer (Waters, Manchester, United Kingdom). The sample was loaded into nanoelectrospray capillaries (Proxeon, Odense, Denmark). Argon was used as the collision gas. Data acquisition and analysis were performed with the reflector W mode for MS and with the V reflector mode for MS-MS using MassLynx, version 4.0 (Waters, Manchester, United Kingdom). A multicharged precursor ion with an m/z value of 1,325.5 [charge state 7 (+7)] was selected for the fragmentation of intact molecular species.

Bacterial strains. Six different strains were used to test antimicrobial activity: *Staphylococcus aureus* ATCC 29740, *Listeria monocytogenes* strain EGD, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* serovar Enteritidis LA5, and *Salmonella enterica* serovar Typhimurium ATCC 14028. *Salmonella enterica* serovar Enteritidis LA5 is a wild-type strain (nalidixic acid resistant at 20 μ g/ml) isolated from natural chicken infections. *Salmonella enterica* serovar Enteritidis ATCC 13076 and *Salmonella enterica* serovar Typhimurium ATCC 14028 were purchased from the Centre de Ressources Biologiques de l'Institut Pasteur (CRBIP, Institut Pasteur, Paris, France). *Staphylococcus aureus* ATCC 29740 was kindly provided by Pascal Rainard (INRA, UR1282, Nouzilly, France), and *Listeria monocytogenes* strain EGD and *Salmonella enterica* serovar Enteritidis LA5 were kindly provided by Philippe Velge (INRA, UR1282, Nouzilly, France).

Antimicrobial activity tests. The antibacterial activities of AvBD11 were measured by radial diffusion assay according to the method described by Lehrer et al. (17). Bacteria incubated overnight were diluted in Trypticase soy broth (TSB) or brain heart infusion (BHI) at an absorbance of 0.02 and were incubated for 2.5 to 4 h, depending on the bacterial strain, at 37°C to obtain a mid-logarithmic-phase culture. Bacterial concentrations were determined by plating serial 10-fold dilutions of bacterial suspension on Trypticase soy agar (TSA) plates and by counting CFU after 24 h of incubation at 37°C. A volume containing bacteria at 1.10^7 CFU was centrifuged at $900 \times g$ for 5 min at 4°C, and the bacteria were

washed once with cold 10 mM sodium phosphate buffer (pH 7.4), resuspended in a small volume of cold sodium phosphate buffer, and mixed with 25 ml of previously autoclaved, warm (42°C) 10 mM phosphate buffer containing 0.03% TSB medium, 1% (wt/vol) low-endosmosis agarose (Sigma-Aldrich, Saint Quentin Fallavier, France), and 0.02% Tween 20. The agarose solution containing the bacteria was poured into a petri dish to form a 1-mm-deep uniform layer. A 2.5-mm-diameter gel punch was used to make 36 evenly spaced wells. In each well, 5 μ l of a peptide dilution or control solution was added. MSI-94 (a linear amphipathic magainin variant displaying a broad antimicrobial spectrum) and AvBD103b (spheniscin-2) were used as positive controls. MSI-94 was a kind gift from Philippe Bulet (CNRS UMR 5525, Archamps, France). AvBD103b was a synthetic peptide with its 3 disulfide bonds intact that was purchased from Genepep (Montpellier, France). The peptides were allowed to diffuse in the bacteria-containing gel while the plates were incubated for 3 h at 37°C. The gel was then overlaid with 25 ml of agar consisting of a double-strength (6% [wt/vol]) solution of TSB containing 1% (wt/vol) agarose. After an overnight incubation at 37°C, the diameter of the inhibition zone around each well was calculated as the diameter in mm of the clearance zone surrounding the well minus the diameter in mm of the well. For each bacterial strain, three identical independent measurements of antibacterial activity were performed. The MIC of each peptide was determined as described by Lehrer et al. (17). The best-fit straight line was determined using linear regression in Microsoft Excel 2003 (Microsoft Office). The MIC was calculated by finding the intersection of the line with the x axis, indicating the lowest peptide concentration at which no clear zone was obtained.

RESULTS

Identification and purification of AvBD11 from vitelline membranes. We explored AvBD11 purification from the vitelline membrane, since this protein was described as a major constituent. Following extraction, heparin-Sepharose chromatography was used as the first step of the purification scheme; proteins that bind to this resin were eluted at a high salt concentration and were analyzed by SDS-PAGE. The fraction containing proteins with affinity to heparin-Sepharose exhibits a wide range of proteins (10 to 100 kDa) with a major band of 10 kDa, corresponding to AvBD11's molecular mass. Western blotting with specific anti-AvBD11 antibodies confirmed its presence and revealed an enrichment of AvBD11 in the fraction eluted from the heparin-Sepharose beads. The corresponding band (about 10 kDa) was excised from the SDS-PAGE gel, and its peptide sequence analyzed by nano LC-ion trap MS. Analysis of the data revealed 3 peptides matching the AvBD11 sequence which was predicted from the *Gallus gallus* genome and 3 peptides corresponding to *Gallus gallus* lysozyme (Table 1). In order to separate AvBD11 from the lysozyme and additional proteins, this fraction that was obtained using heparin-Sepharose chromatography was further purified by RP-HPLC using an X-Bridge C₁₈ column (Fig. 1). The collected fractions were subjected to tryptic digestion, and the resulting peptides were identified using nano LC-Q-TOF MS. Pure AvBD11 without any contaminant was identified in the RP-HPLC fraction collected at 35 min (Fig. 1 and Table 2), and using the NCBI nr database, avian β -defensin 11 (*Gallus gallus* [gi|49169808]) was identified with a Mascot protein score of 514 and a protein sequence coverage of 53.8% (Table 2).

Structural characterization of AvBD11 using a top-down proteomic approach. A top-down mass spectrometry strategy was applied to purified AvBD11 to determine its primary structure at the N and C termini and to characterize posttranslational modifications. An aliquot of intact AvBD11, purified by the chromatographic procedures described above, was subjected to MS and MS-MS analysis using nano ESI-Q-TOF MS without any prior reduction or alkylation steps. The multi-

TABLE 1. Identification by nano LC-Q-TOF mass spectrometry of heparin-binding proteins from egg vitelline membranes, present in the 10-kDa band

GI and accession no. ^a	Protein	Protein Mascot score	Protein mass (Da)	No. of unique peptides	Peptide sequence
gi 49169808, NP_001001779, Q6IV20	Avian β -defensin 11	703	11,642	3	CLEEQLGLCPLK VCPKPFAAFGTCSWR IRCLEEQLGLCPLKR
gi 157831653, 1KXW_A, P00698	Lysozyme C	348	14,305	3	NTDGSTDYGILOINSR NLCNIPCSALLSSDITASVNCAL GYSLGDWVCAAKFESNFNTQATNR

^a NCBI GI no., NCBI protein accession no., and Swiss-Prot TrEMBL protein accession no.

charged ions of the mature AvBD11 were measured with a mass accuracy of 100 ppm, affording m/z values of 1,159.83, 1,325.36, and 1,546.09, respectively, for the (+8), (+7), and (+6) charge states. Through the use of high-resolution data obtained with a Q-TOF mass spectrometer in W reflectron mode, the monoisotopic mass $[M + H]^+$ was calculated and determined at $9,271.56 \pm 0.12$ Da (Fig. 2). In the MS-MS spectrum of the (+7) precursor ion at m/z 1,325, the N-terminal sequence LPRDTSR of mature AvBD11 was determined by six fragment b ions, b_2 to b_7 , and the C-terminal sequence KEI was confirmed by three fragment y ions (y_1 to y_3) (Fig. 3). Altogether, these data suggest that the complete, mature AvBD11 peptide consists of 82 amino acids with the following sequence: LPRDTSRCVGYHGYCIRSKVCPKPFAAFGTCSWRQKTCCVDTTSDFTTCQDKGGHCVSPKIRCLEEQLGLCPLKRWTCKEI. The observed N-terminal sequence LPRDTSR of mature AvBD11 is two amino acids longer than that predicted by *in silico* studies (RDTSR) (31) and corresponds exactly to that predicted by the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP>). In fact, the N-terminal sequence LPRDTSR is identical to that determined by

Kido et al. (12) for VMO-2. Based on its predicted sequence, the theoretical monocharged monoisotopic mass of mature AvBD11 was calculated to be 9,283.37 Da, differing by 12 Da from that determined experimentally (9,271.56 Da), which corresponds to the posttranslational modifications resulting from six disulfide bonds formed from the 12 cysteine residues of AvBD11.

Antibacterial activity of AvBD11. The antimicrobial activities of purified AvBD11, AvBD103b, and MSI-94 were evaluated using a radial diffusion assay against six different pathogens: *Staphylococcus aureus* ATCC 29740, *Listeria monocytogenes* strain EGD, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* serovar Enteritidis LA5, and *Salmonella enterica* serovar Typhimurium ATCC 14028. The inhibition zone (expressed in units [1 mm equal to 1 U]) was calculated as the diameter in mm of the clearance zone surrounding the well minus the diameter in mm of the well. They were further plotted as a \log_{10} function (peptide concentration) for each bacterial strain, as illustrated in Fig. 4 for AvBD11 against *L. monocytogenes*. The various MICs obtained for each peptide are shown in μ M in Table 3. The antimicrobial peptides MSI-94 and AvBD103b were used as controls, and similar MICs were observed for AvBD11 against *S. Enteritidis* ATCC 13076 and *S. Typhimurium*. AvBD11's antibacterial activities toward *S. aureus* were three and five times lower than those of the MSI-94 and AvBD103b controls. However, for *E. coli*, the results showed that AvBD11 and AvBD103b displayed particularly low MICs, 50 and 90 nM, respectively, compared to 370 nM obtained for MSI-94. AvBD11 and AvBD103b also displayed a stronger antibacterial effect than MSI-94 against *L. monocytogenes*. While AvBD11 was two times less effective than AvBD103b against *S. Enteritidis* LA5, their MICs were similar against *S. Enteritidis* ATCC 13076.

DISCUSSION

AvBD11 has been previously identified by proteomic approaches in different compartments of the hen egg—the egg-shell, the egg white, and the vitelline membranes (21–23). In the work reported herein, we have developed a fast, simple, and reliable method to purify AvBD11 from vitelline membranes. The mature AvBD11 peptide was purified from vitelline membranes by a combination of heparin-Sepharose chromatography and C_{18} RP-HPLC. We obtained around 750

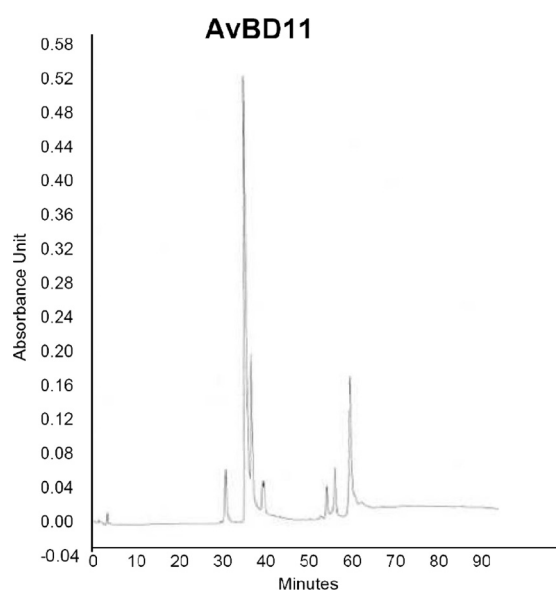


FIG. 1. Reverse-phase HPLC was performed to separate molecules of the vitelline membrane eluted fraction obtained after heparin-Sepharose affinity chromatography.

TABLE 2. Peptide list obtained from reverse-phase HPLC-purified AvBD11 after tryptic reaction and identified in NCBI nr database using data from nano LC-Q-TOF MS^a

Peptide	<i>m/z</i>	Z	M obs (Da)	M theo (Da)	ΔM (Da)	No. of missed cleavages	Mascot score	Position	Modification
YHGYCIR	484.71	2	967.41	967.43	−0.02	0	20.64	33–39	CAM
GYHGYCIR	513.21	2	1024.40	1024.45	−0.06	0	32.90	32–39	CAM
AAFGTCSWR	528.23	2	1054.45	1054.47	−0.02	0	65.46	48–56	CAM
VGYHGYCIR	562.75	2	1123.48	1123.52	−0.04	0	44.09	31–39	CAM
CVGYHGYCIR	642.76	2	1283.50	1283.55	−0.05	0	57.96	30–39	2 CAM
PFAAFGTCSWR	650.29	2	1298.57	1298.59	−0.02	0	86.99	46–56	CAM
EEQLGLCPLKR	671.85	2	1341.68	1341.71	−0.03	1	59.87	87–97	CAM
LEEQLGLCPLKR	728.38	2	1454.75	1454.79	−0.04	1	64.13	86–97	CAM
CLEEQLGLCPLK	730.35	2	1458.69	1458.72	−0.03	0	72.70	85–96	2 CAM
CLEEQLGLCPLKR	539.27	3	1614.79	1614.82	−0.03	1	98.86	85–97	2 CAM
CLEEQLGLCPLKR	808.40	2	1614.79	1614.82	−0.03	1	85.86	85–97	2 CAM
VCPKPFAAFGTCSWR	595.27	3	1782.78	1782.83	−0.05	0	79.60	42–56	2 CAM
TCCVDTTSDFTCQDK	658.90	3	1973.69	1973.76	−0.07	0	64.17	59–74	3 CAM
TCCVDTTSDFTCQDKGG	1016.37	2	2030.73	2030.78	−0.05	1	22.90	59–76	2 CAM

^a *m/z*, mass/charge ratio (*m/z*) at which fragmentation occurred; Z, charge state of ion precursor; M obs, experimentally obtained molecular mass; M theo, theoretical molecular mass; ΔM, difference between theoretical and observed peptide precursor masses; Position, position in the full sequence; Modification, observed carbamidomethylation (CAM). The peptides allowed the identification of β-defensin 11 of *Gallus gallus* (gi49169808) with a Mascot protein score at 514 and 53.8% for the protein coverage.

μg of AvBD11/250 mg of solubilized vitelline membranes (extracted from 30 eggs). The characterization of mature AvBD11 was achieved using a hybrid strategy combining “bottom-up” and “top-down” proteomic approaches, as previously described for AvBD1, AvBD2, and AvBD7 (3). The use of complementary mass spectrometry techniques allowed us to obtain structural information concerning the mature AvBD11 peptide sequence and its posttranslational modifications. The sequence

of mature AvBD11 was shown to be composed of 82 amino acids. The amino-terminal sequence, identified as LPRDTSR, corroborates the previous determination by Kido et al. (12). Top-down proteomic approaches put in evidence the formation of six disulfide bonds from the 12 cysteine residues. We attempted to carry out complementary mass spectrometry analyses but failed to determine the position of the disulfide bonds. These results are partly explained by the high resistance

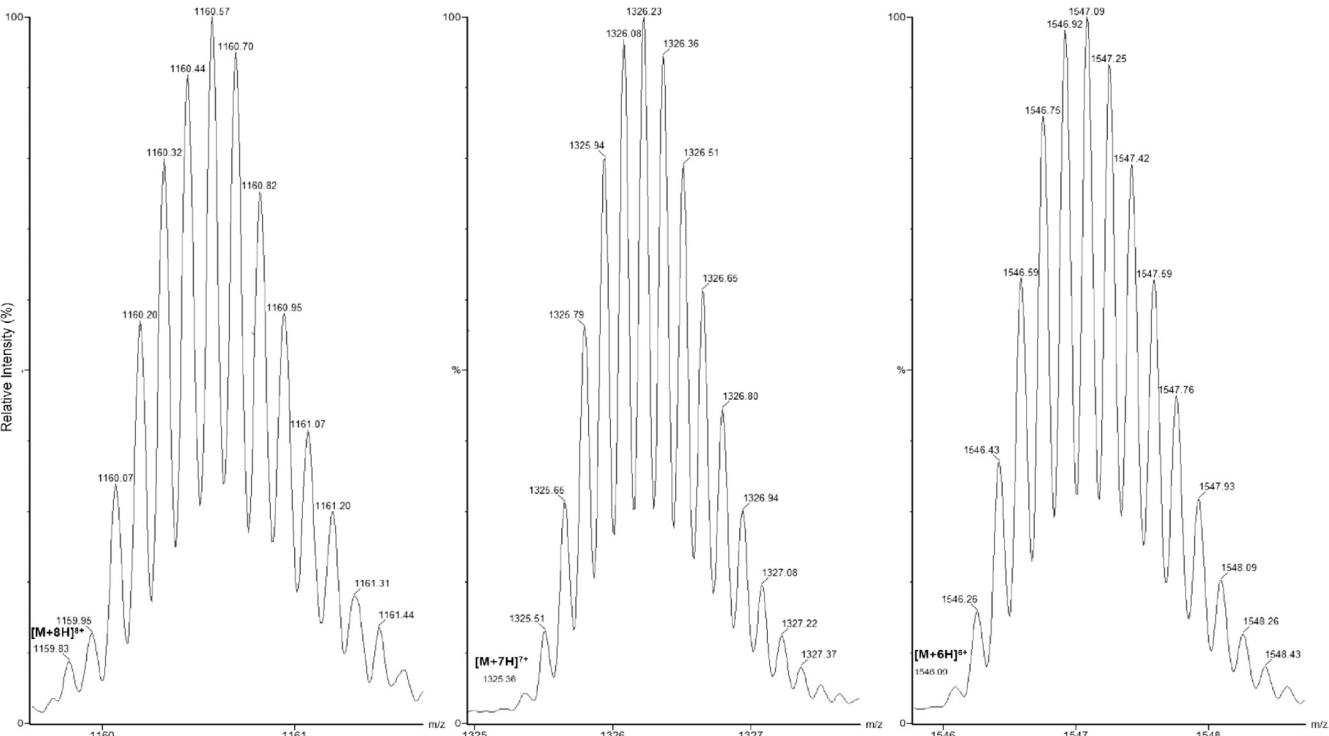


FIG. 2. Multicharged-ion spectra for intact AvBD11. Spectra (zoomed on multicharged species) were obtained with nano ESI-Q-TOF MS with reflector W mode (resolution *R* = 15,000) from intact AvBD11 purified by reverse-phase HPLC. The monoisotopic molecular mass of the mature AvBD11 was observed at *m/z* 1,159.83, 1,325.36, and 1,546.09, respectively, for the (+8), (+7), and (+6) charge states. The monocharged molecular species [M + H]⁺ was calculated and determined at 9,271.56 ± 0.12 Da.

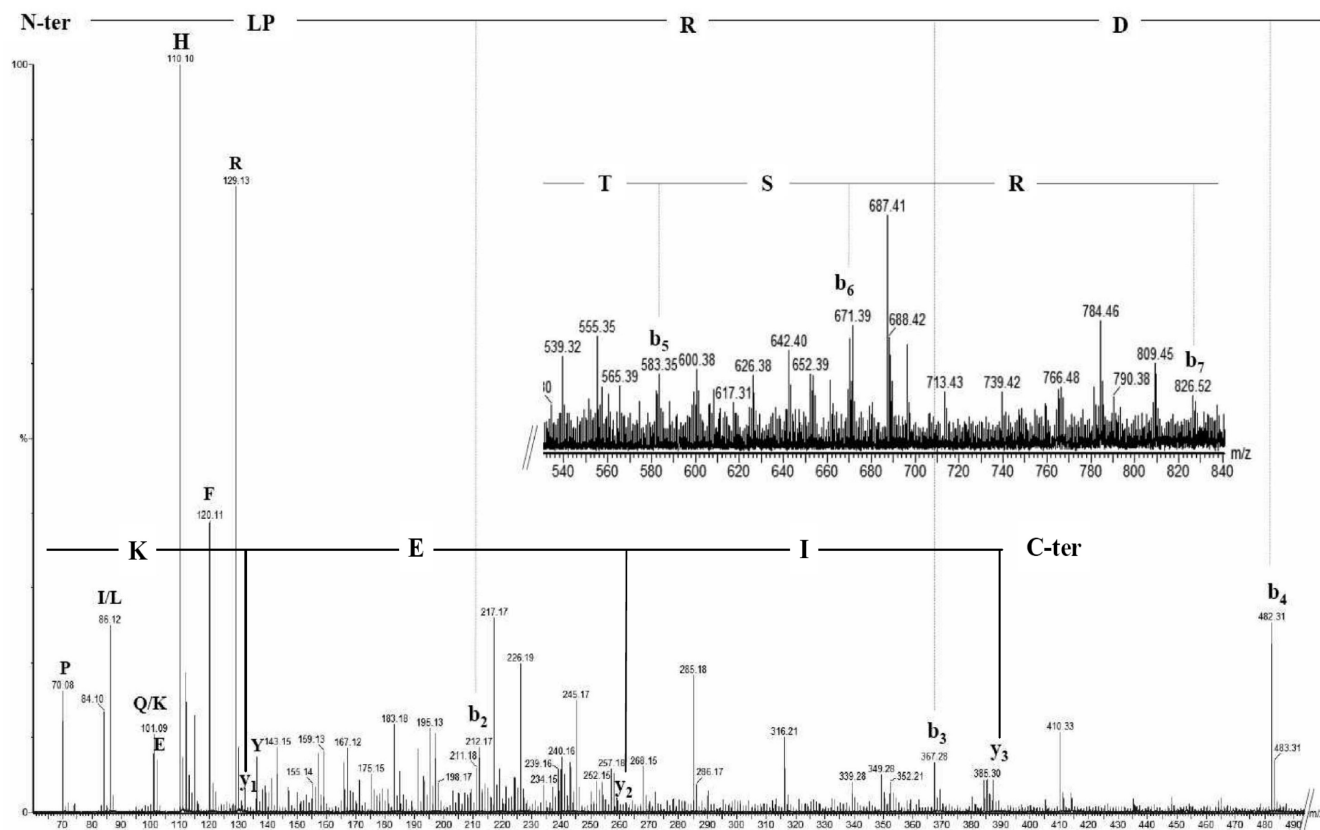


FIG. 3. C and N termini of the sequence of AvBD11 extracted from vitelline membrane. Shown is an MS-MS spectrum obtained by collision-induced dissociation with nano ESI-Q-TOF MS from intact AvBD11 without prior reduction or alkylation. The precursor ion had an m/z of 1,325.5 in charge state 7. The N-terminal sequence LPRDTSR, determined by manual interpretation, was essentially obtained by six consecutive b ions (b_2 to b_7), and the C-terminal sequence KEI was confirmed by three y ions (y_1 to y_3). Single letters indicate immonium ions.

of AvBD11 to tryptic digestion and to heat denaturation. The strong stability of mature AvBD11 is compatible with the prediction of six internal disulfide bonds. The entire AvBD11 sequence has no homology with any other β -defensin. To our knowledge, AvBD11 is the first β -defensin containing a double β -defensin motif with 6 disulfide bonds to be purified and characterized. Its C-terminal sequence possesses 27% identity to the corresponding region of human β -defensin 3 (Swiss-Prot accession no. P81534), which seems to include the biologically

active part of the molecule (14). Solving the three-dimensional structure by two-dimensional nuclear magnetic resonance and molecular modeling is needed in order to determine the pairing pattern of the six disulfide bridges and to understand the structure-function relationship of AvBD11, as has been performed for AvBD103b (15).

Avian β -defensins interact electrostatically with negatively charged components of bacterial membranes, such as lipopolysaccharides, lipoteichoic acid, and anionic phospholipids. De-

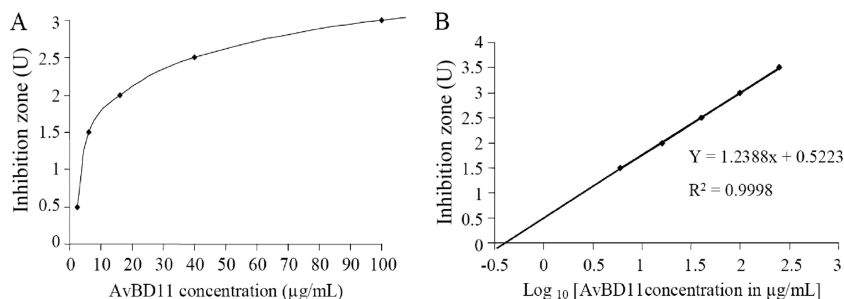


FIG. 4. AvBD11 antimicrobial activity against *Listeria monocytogenes*. (A) Dose-response of AvBD11 and its inhibitory effect on *L. monocytogenes*, reflected by the size of the inhibition zone (expressed in units, with 1 U equal to 1 mm, and calculated as the diameter in mm of the clear zone on the plate minus the diameter in mm of the well). (B) The best-fit straight line was determined by linear regression. The MIC of AvBD11 against *L. monocytogenes* was calculated by finding the intersection of the line with the x axis, indicating the lowest peptide concentration at which no clear zone was detected.

TABLE 3. MICs of purified AvBD11, synthesized king penguin AvBD103b, and MSI-94

Bacterial group, species	MIC ^a (μ M) (95% confidence interval)		
	MSI-94 ^b	AvBD11	AvBD103b
Gram positive			
<i>S. aureus</i> ATCC 29740	0.33 (0.19–0.48)	0.90 (0.27–1.7)	0.16 (0.12–0.20)
<i>L. monocytogenes</i>	0.28 (0.13–0.43)	0.18 (0.08–0.27)	0.14 (0.14–0.15)
Gram negative			
<i>S. Enteritidis</i> ATCC 13076	0.31 (0.25–0.35)	0.35 (0.27–0.46)	0.31 (0.15–0.45)
<i>S. Enteritidis</i> LA5	0.15 (0.10–0.21)	0.40 (0.29–0.49)	0.21 (0.20–0.22)
<i>S. Typhimurium</i> ATCC 14028	0.25 (0.11–0.40)	0.32 (0.31–0.32)	0.20 (0.19–0.21)
<i>E. coli</i> ATCC 25922	0.37 (0.23–0.52)	0.05 (0.04–0.05)	0.09 (0.08–0.11)

^a The MIC was determined by radial diffusion assay for each bacterial strain.

^b MSI-94 is a magainin variant used as a positive control.

pending on their amino acid composition, AvBDs exhibit more or less antimicrobial activity against a specific bacteria strain (30). In our study, we found that both AvBD11 and AvBD103b exhibited potent antimicrobial activity against the six bacterial strains tested. *S. Enteritidis* LA5 corresponds to a field-isolated strain and, because of the high incidence of *S. Enteritidis* contamination of hen eggs, any antimicrobial peptide that is effective against this particular strain is of interest.

In this study, AvBD11 and AvBD103b possess elevated cationic net charges of (+9) and (+10), respectively. And even though AvBD103b was more cationic than AvBD11, similar levels of antibacterial activity were found for AvBD11 and AvBD103b toward *L. monocytogenes*, *S. Enteritidis*, *S. Typhimurium*, and *E. coli*. We noted that AvBD103b possessed better antibacterial activity than AvBD11 against *S. aureus*. Derache et al. (3) compared the antibacterial activities of AvBD2 (+4), AvBD1 (+8), and AvBD7 (+6). In accordance with our findings, they could not establish a direct link between the net charge of the peptides and their antibacterial potency. In our study, an identical antibacterial assay and positive control (MSI-94), as in the study by Derache et al. (3), were used to characterize AvBD11 and AvBD103b. Therefore, the MICs obtained for AvBD1, -7, -11, and -103b could be compared. Even though AvBD11 and AvBD103b were positively charged at a higher level than AvBD1 and -7, they did not exhibit better antimicrobial activities toward *Salmonella* strains and *S. aureus*, but they were more efficient against *E. coli* (3). These comparisons, taken together, reinforce the fact that no direct relationship could be established between the net charge of these AvBDs and their antimicrobial potency against Gram-positive and -negative bacteria. They also suggest that AvBD's efficiency depends not only on its amino acid composition but also certainly on the positioning of the amino acids. However, it is noteworthy that the relationship between the high positive net charge (+10) of AvBD103b and its potent antimicrobial activity is evident when tested in a salt-rich environment (15).

The innate immune system is known to play a major role in protecting the female reproductive tract against microbes. Previous studies have shown the presence of a large panel of AvBDs in the different regions of the hen oviduct, presumably exhibiting a large spectrum of antimicrobial activities in order to protect the future embryo against pathogenic microorganisms. In humans, during late pregnancy and delivery, human β -defensin 3 (hBD3), which possesses homology with AvBD11, is expressed at key sites of the uterus and would provide en-

hanced protection from infection for the uterus and/or fetus (13). In hens, the onset of egg-laying activity takes place at approximately 5 months of age. AvBD1, -2, and -3 mRNAs are not expressed in the immature oviduct of younger hens or in the mature oviduct of nonlaying hens, but their oviduct expression increases when the hens are forming eggs (25, 34). These results suggest modulation of AvBD expression as a function of egg-laying activity, possibly as a consequence of the dilation of the oviduct by the presence of the forming egg, as shown for osteopontin and ovocalyxin 36 (7, 16). Furthermore, several studies have shown a modulation of β -defensin expression correlating with stimulation by pathogens. In the amnion FL cell line, hBD3 expression increases in response to peptidoglycans and lipopolysaccharide (LPS), which mimic the effect of bacterial infection (2). Modification of AvBD expression has also been demonstrated in the hen oviduct. AvBD1, -2, and -3 mRNA expression was upregulated when cultured hen vaginal cells were stimulated with LPS or with *S. Enteritidis* (33). In the hen vagina, the expression of AvBD3, -5, -10, -11, and -12 were significantly increased in response to LPS treatment (20). In primary hen isthmus epithelial cells, an infection with wild-type *S. Enteritidis* decreased the expression of AvBD4, -9, -10, and -11, which are constitutively highly expressed, and conversely increased the normally low expression of AvBD2, -7, and -8 (4). This mechanism might be present in other regions of the hen oviduct, in order to ensure an appropriate antimicrobial response in case of infection (24). Moreover, in humans, several studies have reported chemotaxis of hBD3 for monocytes and macrophages (32), allowing the recruitment of these cells into uterine tissues. This suggests that hBD3, produced in the uterine tissues, may have a role in the activation of the maternal adaptive immune system in the event of infection (13). In the hen oviduct, AvBD11 might possess similar functions and interact with the adaptive immune system, in addition to its innate molecular antimicrobial activities.

The presence of AvBD11 in different compartments of the egg (eggshell, egg white, and the vitelline membranes) suggests that it plays a protective role during embryonic development. If there are changes in AvBD11's stability during egg storage, this might explain the decreased protective efficiency of the vitelline membrane which is noted during egg storage at 20°C (27). Lysozyme, VMO-1, and VMO-2 (AvBD11) are the three major proteins of the salt-soluble fraction of vitelline membranes. During 20 days of egg storage at 5°C or 20°C, the lysozyme content increases in the salt-soluble fraction of vitelline membranes, while AvBD11 de-

creases from 5% (initially observed) to 3% and 1% at 5°C and 20°C, respectively. The authors attributed the deterioration of vitelline membranes during storage to the disappearance of the VMO-1 and AvBD11 proteins. However, an alternative hypothesis is that the breakdown of the vitelline membranes was due to denaturation of ovomucin, the major component of the vitelline membrane, which is not present in the salt-soluble fraction. In any event, the loss of the vitelline membrane and its components is likely to alter the antimicrobial protection in these compartments during storage.

The current study put in evidence the large antibacterial spectrum of AvBD11, a β -defensin with an unusual structure containing two repeated β -defensin consensus motifs consisting of 6 disulfide bonds. The structural characterization of AvBD11 is expected to lead to superior modeling for the design of synthetic antimicrobial peptides. AvBD11 is present in different compartments of the egg, including the egg white. The availability of antibodies against this peptide will permit the development of a quantitative assay aiming to evaluate the variability of AvBD11 in table eggs, as a function of hen physiological stage (sexual maturity and age) and/or environment. Finally, this work contributes to a broader understanding of how previously unknown egg components contribute to the numerous biological functions of the egg, including its antimicrobial activities (24). Such studies underline the potential of the table egg as a source of new antimicrobial peptides and its value for human and animal health.

ACKNOWLEDGMENTS

We are grateful to Aurélien Brionne, Magali Bergès, and Jean-Claude Poirier for their excellent technical assistance. We thank the staff of the PEAT (Poultry Experimental Unit, INRA, UE609 Unité Avicole, F-37380 Nouzilly, France) and, more particularly, Jean-Didier Terlot-Bryssine for the care of the birds. We thank the staff of the PFIE (Plate-Forme d'Infectiologie Expérimentale, INRA, F-37380 Nouzilly, France) and, especially, Sébastien Lavillatte for the care of the rabbits and his technical support. We also thank Maxwell T. Hincke (University of Ottawa, Canada) for his critical and careful reading of the manuscript and his constructive remarks.

This work has been supported by a grant from the Reducing Egg Susceptibility to Contamination in Avian Production in Europe (RESCAPE) project of the European Commission (FOOD-CT-2006-036018).

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